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Purine Biosynthetic Genes Are Required for Cadmium Tolerance in *Schizosaccharomyces pombe*

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Phytochelatins (PCs) are metal-chelating peptides produced in plants and some fungi in response to heavy metal exposure. A Cd-sensitive mutant of the fission yeast *Schizosaccharomyces pombe*, defective in production of a PC-Cd-sulfide complex essential for metal tolerance, was found to harbor mutations in specific genes of the purine biosynthetic pathway. Genetic analysis of the link between metal complex accumulation and purine biosynthesis enzymes revealed that genetic lesions blocking two segments of the pathway, before and after the IMP branchpoint, are required to produce the Cd-sensitive phenotype. The biochemical functions of these two segments of the pathway are similar, and a model based on the alternate use of a sulfur analog substrate is presented. The novel participation of purine biosynthesis enzymes in the conversion of the PC-Cd complex to the PC-Cd-sulfide complex in the fission yeast raises an intriguing possibility that these same enzymes might have a role in sulfur metabolism in the fission yeast *S. pombe*, and perhaps in other biological systems.

Heavy metals, a group of elements including Cd, Cu, Zn, Bi, Ni, Hg, and Pb, are toxic to biological organisms at various concentrations; some are essential nutrients (e.g., Cu and Zn), but many have no known biological function. Heavy metals released into the environment each year result in toxicity assessed to exceed that from organic chemicals (25). For chelation of heavy metals, fungi and higher eukaryotic organisms have various systems which fall into two main categories. Animal cells and some fungi, including the budding yeast *Saccharomyces cerevisiae*, synthesize metal-chelating proteins called metallothioneins (11), while the fission yeast *Schizosaccharomyces pombe* and plants synthesize enzymatically produced peptides that form chelation complexes with metal ions. The metal-binding peptides produced by plants, algae, and *S. pombe* have been referred to by various investigators as cadystins (16), phytochelatins (9), poly(γ-EC)G (13), Cd-binding peptides (30), and γ-glutamyl peptides (19). To conform to the term used in recent reviews (29, 37), they will be referred to as phytochelatins (PCs).

PCs were first discovered in *S. pombe* by Murasugi et al. (21, 22) as components of metal-containing complexes from Cd-induced cells. Grill et al. (9) subsequently demonstrated that PCs are produced by all higher plants tested (7). PCs have also been shown to exist in the alga *Chlorella fusca* (6) and in the yeast *Candida glabrata* (18, 19). Kondo et al. (14, 15) and Grill et al. (9) described the structure of PCs as consisting of repeating units of γ-glutamylcysteine followed by a C-terminal glycine, with the number of repeating units ranging from 2 to 11. The role of glutathione (GSH) as a precursor in PC synthesis was confirmed by the purification of plant PC synthase, which removes a γ-glutamylcysteine moiety from one molecule of GSH and adds it to another GSH or PC molecule (8). PC synthase was purified from a variety of different plant species and was found to be produced constitutively in the absence of metal induction. Activity of the enzyme was dependent on the presence of heavy-metal ion, and addition of EDTA or metal-free PCs caused PC synthesis to cease (17).

Recently, a second means of PC biosynthesis has been demonstrated in vitro. Using crude extracts prepared from *S. pombe*, Hayashi et al. (12) reported that $(\gamma\text{-Glu-Cys})_{n+1}$ polymers derived from GSH and $(\gamma\text{-Glu-Cys})_n$ formed at low concentrations of GSH. Free glycine was then added to the chain to make the PC peptide in a reaction catalyzed by GSH synthetase. This process occurred in the absence of added heavy-metal ions, and it was not determined whether this pathway is responsible for a significant fraction of PC synthesis in vivo.

In addition to the PC-metal complex isolated from *S. pombe* by Murasugi et al. (22), the authors later described a second complex from the same organism characterized by a higher apparent molecular mass (ca. 10 kDa) and composed of PCs, Cd, and acid-labile sulfide (23). This complex, referred to here as the high-molecular-weight (HMW) PC-Cd-S^{2-} complex, was essential for metal tolerance; several mutant strains deficient in production of the HMW complex were hypersensitive to Cd (24). The requirement for this additional form may be due to its altered properties relative to the low-molecular-weight (LMW) PC-Cd complex, including increased acid stability and an enhanced affinity for metals (32). Of particular interest is the structure of this PC-Cd-S^{2-} complex, which is a CdS quantum semiconductor crystallite surrounded by PCs (3). The PC-Cd-S^{2-} complex, reported also for *C. glabrata* (19), has been described only recently for tomato (*Lycopersicon esculentum*) (31) and for a wild mustard (*Brassica juncea*) that is highly tolerant of selenium (36). In addition, metal-tolerant varieties of *Silene vulgaris* were found to incorporate higher levels of sulfide in the PC-Cd complex (38).

In this paper, we describe the analysis of an *S. pombe* mutant which is deficient in production of the HMW PC-Cd-S^{2-} complex. The analysis of this mutant led to the cloning of the adenylosuccinate synthetase gene and the subsequent

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TABLE 1. *S. pombe* strains

Strain	Genotype	Source
Sp223	h⁻ *leu1.32 ura4.294 ade6.216*	D. Levin
LK69	h⁻ *leu1.32 ura4.294 ade6.216 ade2*	This study (from Sp223)
DS1	h⁻ *leu1.32 ura4.294 ade6.216 ade2::URA3*	This study (from Sp223)
Sp806	h⁻ *ura4-D18*	D. Beach
DS3	h⁻ *ura4-D18 ade2::URA3*	This study (from Sp806)
B1048	h⁺ *ura4.294 ade7.50*	P. Munz
DS5	h⁺ *ura4.294 ade7.50 ade2::URA3*	This study (from B1048)
B23	h⁺ *ade8.106*	P. Munz
CRY302	h⁻ *leu1.32 ura4.294 ade6.216 tub::URA3*	This laboratory (from Sp223)

conclusion that specific steps of the de novo purine biosynthesis pathway are required for conversion from the LMW PC-Cd complex to the sulfide-containing form. A model based on alternate utilization of the purine biosynthesis pathway for the transfer of sulfide from cysteine to the PC-Cd-S^{2-} complex is proposed.

MATERIALS AND METHODS

***Escherichia coli* strains.** ES4 (*tonA2 lacY1* [or *lacZ4*] *tsx-1* [or *tsx-70*] *supE44 gal-6* λ⁻ *mtl-2 purA45*) was obtained from the *E. coli* Genetic Stock Center.

Growth and maintenance of *S. pombe* strains. *S. pombe* strains, listed in Table 1, were cultured at 30°C on either minimal medium SD (6.7 g of yeast nitrogen base [Difco], 20 g of glucose per liter) or rich medium YG (5 g of yeast extract [Difco], 20 g of glucose per liter). Where appropriate, nutritional supplements were added at 20 μg/ml for nucleotide bases and 100 μg/ml for amino acids. For solid media, Select agar (Gibco) was added to 1.5%.

Isolation of mutants. Sp223 was treated with ethyl methanesulfonate to produce approximately 90% killing. The cells were washed with 5% $NaSSO_3$ and plated on YG agar. Individual mutagenized clones were screened for sensitivity to $CdCl_2$ by individual patching onto YG media containing various concentrations (0, 100, 200, and 400 μM) of $CdCl_2$. Isolates which appeared to be hypersensitive were checked to ensure the absence of additional auxotrophic requirements prior to gel filtration analysis to determine whether they displayed altered PC accumulation.

Gel filtration analysis. Cells were grown to an optical density at 595 nm of 0.4 in YG (or SD for strains with plasmids), and then $CdCl_2$ was added to a final concentration of 200 μM. After 30 h of induction (except where stated), the cells were harvested by centrifugation at 2,200 × *g* for 5 min, washed twice with 50 mM Tris (pH 7.8) at 0°C, and resuspended in 0.5 to 1% of the original volume of the same buffer. Cells were broken by vortexing with glass beads with five 30-s bursts followed by 1-min rests on ice. Cell breakage was monitored by phase-contrast microscopy, and extracts were cleared by centrifugation for 5 min at 15,000 × *g*. Two milligrams of protein, as determined by the dye binding assay (Bio-Rad Laboratories), was adjusted to 1 ml and loaded onto a Sephadex G-50 column (equilibrated with 50 mM Tris-Cl [pH 7.8]). Sample buffer was 5% glycerol–10 mM dithiothreitol. The column was developed at 50 ml/h, and 2-ml fractions were collected. PCs were labeled through the inclusion of 0.25 μCi of ^{109}Cd (NEN; 0.4 Ci/mmol) in the applied sample. PC-containing peaks were identified by counting 1 ml of each fraction in a liquid scintillation counter.

Isolation of genomic and cDNA clones. The genomic and cDNA libraries used in this study (26), DNA transformation (2), and recovery of complementing plasmids in *E. coli* JA226 (1) were described previously. For cloning of the cDNA, a phage λgt10 cDNA library was screened with the 2-kb genomic DNA insert of pDH4. Ten hybridizing plaques were identified and purified from a total of 30,000 screened. Phage DNA was prepared from six of these plaques, cleaved with *Eco*RI or *Not*I (restriction sites present in the adaptors used to ligate the cDNA into the λgt10 vector), and analyzed by Southern blot hybridization.

Northern (RNA) blot hybridization. Poly(A)⁺ RNA was subjected to agarose gel electrophoresis in the presence of formaldehyde and then transferred to a nylon membrane (MSI). The blot was hybridized in the presence of 50% formamide to an [α-^{32}P]dCTP-labeled probe derived from pDH4 prepared as described previously (4).

DNA sequencing and analysis. The DNA sequences of the genomic and cDNA inserts were determined by the method of Sanger et al. (33) by using double-stranded templates and 17-nucleotide (nt) primers complementary to the newly determined sequence. The DNA sequences were analyzed by using the Intelligenetics program, and the deduced amino acid sequences were compared with the Swiss-Prot data base.

***S. pombe* chromosome isolation.** *S. pombe* chromosomes were isolated from lysed spheroplasts and separated by pulsed-field gel electrophoresis by the method of Smith et al. (35). DNA from each of the chromosomal bands was purified from the gel by using Geneclean (Bio 101), digested with *Bam*HI and *Pst*I, and then subjected to Southern blot hybridization analysis with an α-^{32}P-labeled DNA probe derived from pGS1 insert DNA.

Gene disruption and Southern blot analysis. Gene disruption was accomplished by transformation of spheroplasts with a linear DNA fragment (see Fig. 6). Following selection for the *S. cerevisiae URA3* marker, transformed clones were screened by Southern blot hybridization. Chromosomal DNA prepared as described previously (26) was cleaved by restriction endonucleases, fractionated by agarose gel electrophoresis, blotted to Zetaprobe transfer membrane (Bio-Rad), and hybridized with α-^{32}P-labeled DNA probes.

Sulfide analysis. Acid-labile sulfide was determined by a modification of the method described by Rabinowitz (28). After reaching an optical density of 0.4, a 100-ml YG-grown culture of each strain was split into two 50-ml portions. To one of these was added $CdCl_2$ to a final concentration of 200 μM. Each culture was incubated at 30°C in a shaking water bath in a sidearm flask bearing a rubber stopper through which a glass pipet was inserted. The external end of the pipet was attached to a sterilized air filter, through which humidified air was supplied by bubbling through H_2O. The internal portion of the pipet was submerged below the

surface of the culture medium to aerate the culture. The side arm was connected by autoclaved tubing to a Pasteur pipet whose tip was submerged in 1 M zinc acetate (ZnOAc) contained in a tube taped to the side of the flask, allowing the ZnOAc to trap the volatile sulfide released by the culture. After 24 h of induction, each culture was removed, 1 ml was set aside, and the cells were harvested by centrifugation. The 50-ml supernatant was returned to the sidearm flask (with the cell pellets stored on ice), 10 ml of concentrated HCl was added, and the aeration and shaking were resumed for 30 min. At this point, the acidified medium was removed and the ZnOAc was replaced with fresh ZnOAc in new tubes. The cell pellets were resuspended in 50 ml of fresh YG medium and returned to the sidearm assemblies along with 10 ml of concentrated HCl and 0.5 ml of toluene. The assemblies were shaken with aeration for 40 min, after which the ZnOAc tubes were removed for assay. In order to provide a normalization factor between the cultures, the 1 ml of culture reserved was centrifuged in preweighed microcentrifuge tubes. The cell pellets were saved and dried under vacuum and then weighed to determine dry weight per milliliter. The trapped sulfide was assayed by addition of either 0.25 (plus 0.25 ml of fresh ZnOAc) or 0.5 ml of the ZnOAc trap solution to 0.7 ml of H_2O. To each tube was added the following: (i) 0.1 ml of 6% NaOH, followed by vigorous vortexing; (ii) 0.25 ml of *N,N*-dimethyl-*p*-phenylene diamine HCl (0.1% in 6 N HCl), followed by vortexing; and (iii) 0.1 ml of $FeCl_3$ (0.31% in 0.6 N HCl), with a final vortex. After 30 min, the A_{670} of each sample was measured.

Nucleotide sequence accession number. The GenBank accession number for the *S. pombe ade2* gene is M98805.

RESULTS

A Cd-hypersensitive mutant deficient in the production of the PC-Cd-sulfide complex. *S. pombe* Sp223 (Table 1) is able to grow in the presence of Cd concentrations of up to 1 mM. Sp223 was mutagenized with ethyl methanesulfonate, and isolates which were found to be hypersensitive to Cd (i.e., unable to grow at concentrations as low as 100 μM) were tested for newly acquired auxotrophic requirements. Cd-sensitive mutants without additional growth requirements were tested for their ability to produce PCs by the gel filtration assay. The gel filtration profile of one such mutant, LK69, is shown compared with that obtained from Sp223 (Fig. 1A). LK69 produces normal levels of the LMW PC-Cd complex but little or no HMW PC-Cd-S^{2-} complex. In this way, LK69 is similar to the mutants described by Mutoh and Hayashi (24), showing that formation of the LMW PC-Cd complex is not sufficient to yield a wild-type level of Cd tolerance.

Identification of the adenylosuccinate synthetase gene. The gene affected by the mutation in LK69 was isolated by transformation of LK69 with a plasmid library containing genomic DNA from Sp223. The Cd tolerance of LK69 was restored to that of Sp223 by a plasmid clone containing an insert of approximately 10 kb of *S. pombe* genomic DNA. This plasmid, pGS1, also restored accumulation of the HMW PC-Cd-S^{2-} complex to LK69, but its presence in Sp223 enhanced neither Cd tolerance nor HMW complex accumulation (not shown). From a series of deletion subclones, the region of complementing DNA was found to reside in a 2-kb portion lying at one end of the original insert.

The insert of pDH4, the plasmid which contains the smallest defined complementing region, was used as a probe to examine the pattern of expression of the gene at the level of mRNA (Fig. 2). A Northern blot containing mRNA from cells grown in the presence and absence of $CdCl_2$ revealed that there was a single main message with a length of 1.5 kb. There was no significant increase in the level of mRNA upon exposure to Cd. In addition, there was a second visible band of approximately 1.9 kb which corresponds to the predicted length of the unspliced RNA transcript (see below).

A cDNA library constructed from poly(A)$^+$ mRNA isolated from cells grown in the presence and absence of $CdCl_2$ was probed with the complementing sequences present in pDH4. The cDNA insert from a hybridizing λ clone was subcloned into pUC19 and its DNA sequence, along with the genomic sequence in pDH4, was determined. The cDNA clone had a length of 1,493 bp, consistent with the observed size of the message. The comparison of the cDNA and genomic sequences revealed the presence of two introns and a complete open reading frame of 434 amino acids (Fig. 3). Comparison of the deduced protein sequence with those in the Swiss-Prot data base resulted in identification of three highly similar proteins, all identified as the enzyme adenylosuccinate synthetase (Fig. 4) (10, 40, 41). Two of the enzymes, from *Dictyostelium discoideum* and *Mus musculus*, exhibit greater than 50% identity with the predicted product of the cloned gene. On the basis of this analysis, the cloned gene was preliminarily identified as the *ade2* gene from *S. pombe*. Subcloning of the cDNA into the pART1 vector and transformation of LK69 resulted in restoration of Cd tolerance and HMW PC-Cd-S^{2-} accumulation (not shown).

The *ade2* gene in *S. pombe* is known to be located on chromosome I. To verify that the cloned DNA sequences are present on the correct chromosome, *S. pombe* chromosomes were separated and isolated. A Southern blot hybridization using genomic sequences from pGS1 as a probe verified that these sequences hybridized exclusively to chromosome I DNA (Fig. 5). The cDNA was also subcloned into the *E. coli* vector pSK to yield pDH34, which was then transformed into ES4, an *E. coli* strain carrying a mutation in *purA*, the adenylosuccinate synthetase gene. ES4 containing pDH34 was able to grow on minimal medium lacking adenine, whereas ES4 containing pSK required adenine supplementation; similar results were obtained with an *S. pombe ade2* mutant tester strain (not shown). All available data are consistent with the identification of the cloned gene as encoding adenylosuccinate synthetase. Thus, the mutation in LK69 results in deactivation of the *ade2*$^+$ gene, forming an *ade2 ade6* double mutant; the phenotype of this mutation was not revealed during the auxotroph screening process because the *ade6.216* allele in Sp223 necessitated the supplementation of adenine to its derivative strains.

Phenotype of an engineered deletion. To determine whether a deficiency in adenylosuccinate synthetase in an otherwise *ade*$^+$ background would reproduce the mutant phenotype, we engineered a gene disruption event in the chromosome of Sp806 (Table 1) to create a defined deletion in *ade2* (Fig. 6A). The *Bst*EII-*Cla*I fragment of pDS9 was replaced by a 2.0-kb fragment containing the *URA3* gene from *S. cerevisiae*. Linear DNA containing this marker flanked by outlying regions of *S. pombe* chromosomal DNA was then transformed into Sp806 (Table 1) to yield strain DS3. Structure of the disrupted region in DS3 was verified by Southern blot hybridization analysis (Fig. 6B) of chromosomal DNA from the transformed strain and from CRY302 (Table 1), a strain containing a *URA3* integration elsewhere in the genome. DS3 was found to require adenine, demonstrating that the cloned *ade2* DNA represents a single-copy gene; unlike

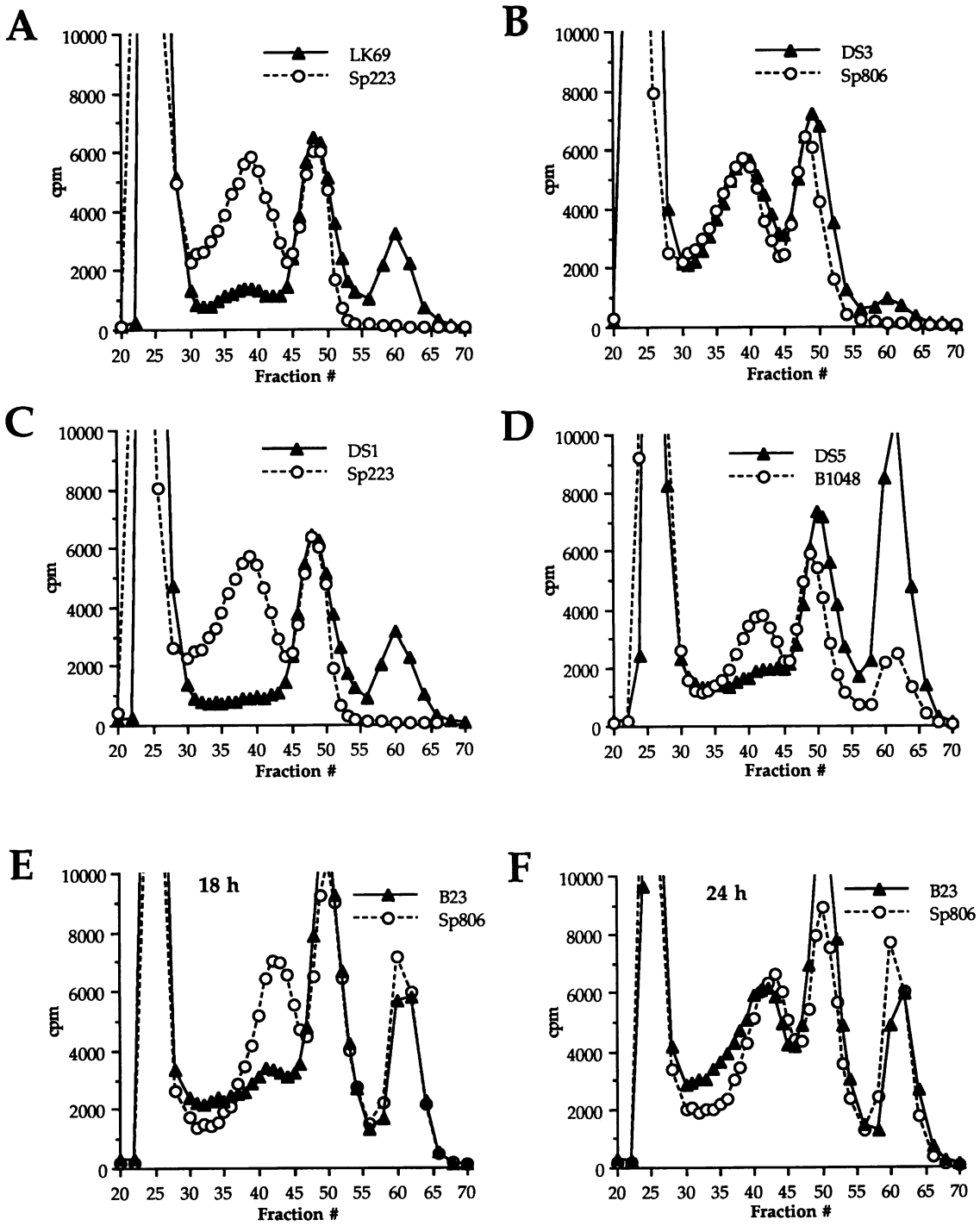

FIG. 1. Gel filtration analyses of cell extracts. Two milligrams of protein from each extract was combined with 0.25 μCi of ^{109}Cd and run on a Sephadex G-50 column; ^{109}Cd in the indicated fractions was detected by liquid scintillation counting. The large void volume peak centered at fraction 25 is due to nonspecific binding of Cd to cell wall fragments and nucleic acids; the HMW PC-Cd-S^{2-} peak is centered around fraction 40, the LMW PC-Cd peak is centered around fraction 49, and the free Cd peak is centered around fraction 60. (A) Cd-sensitive mutant LK69 and its parent Sp223; (B) engineered *ade2* mutant strain DS3 and its parent Sp806; (C) *ade2 ade6* mutant strain DS1 compared with its *ade6* progenitor Sp223; (D) the *ade2 ade7* mutant DS5 and its *ade7* mutant parent, B1048; (E and F) lower rate of accumulation of the HMW PC-Cd-S^{2-} complex in the *ade8* mutant B23 than in Sp806 as seen in extracts from cultures induced with $CdCl_2$ for 18 (E) or 24 (F) h.

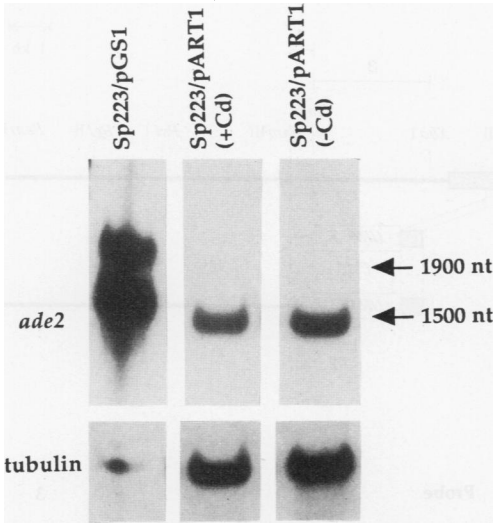

FIG. 2. The cloned gene is not regulated by exposure to Cd. Northern blot analysis of poly(A)$^+$ RNA demonstrates that the level of mRNA which hybridizes to the complementing gene does not change upon exposure to Cd [Sp223/pART1 (+Cd) versus Sp223/pART1 (−Cd)]. The presence of pGS1 increases the total level of hybridizing mRNA, as well as allowing visualization of a second species of RNA (1,900 nt) corresponding to the expected size of the unspliced transcript. The same blot hybridized to a tubulin DNA probe demonstrates that the amounts of RNA loaded in the Sp223/pART1 lanes are equivalent.

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  1-ATCACCAAAACGGAGATCTAATAAAAACATAGAAGAAAAATATGGCTTCAGTGCGAGAAA
 61-CTGGTGTAAATGTTTCCAATGATGGGTAAGAAAATTCTTTGAGAAGAATTTACTATTCAC
121-ACAGAATAACCGTCGTCCTTGGCAGTCAATGGGGTGACGAAGGAAAGGGTAAACTTGTTG
181-ATATCCTTTGCGATAATGTTGATGTCTGTGCTCGCTGTCAGGTATGTTACAAAAGCTTTA
241-TATAGTGGTGAACCGAACGAGGTGATATTGTGTAAAATTTTTTTTCACTCTACTTTGACT
301-CTTAAGTGCGGGACGTCGCGGAATTACTGCCGATGAAAAATAACATTTTTAATAAAAAAT
361-TTAATTTTTTGTTTAAATGTTAATACAAATCTGCTAAATGACTTTTCCTACTTGGTACAG
421-TCAAGAAAAGTATTTTAGAGCTCACTGGATAAGACGATCTGCATCAGTAACAAATAAATT
481-TATACCAATTCTGAATGGTTTCTTCTTTTTGATTTAATTATGGAGGTTACCTTTATGATC
541-TTGCCAGCTTTTTTGTTTGCCCTTGTATTGATTTTTCAAATATATAGCTAACTTTTATTT
601-TTAGGGTGGTAACAATGCTGGTCATACCATTGTTGCTAATGGTGTCACTTACGATTTTCA
661-CATTCTTCCTTCAGGACTCGTAAATCCTAAATGCCAGAACTTGATTGGTTCTGGTGTTGT
721-CGTCTATTTACCTGCTTTTTTTAGTGAGTTAGAGAAACTTGTGCAAAAGGGTTTGAAATG
781-TAGAGATCGTCTTTTCATTTCTGATCGCGCTCATCTAGTATTTGACTACCATCAACGTGC
841-AGATGCTTTAAACGAGGCAGAGCTCGGAAAACAAAGCATCGGAACAACCGGAAAGGGTAT
901-TGGTCCTGCTTATTCCACCAAGGCTACTCGCAGCGGTATTCGTGTTCATCACTTGTATCA
961-TTGGGCTGAATTTGAAGCTCGTTACCGTAAGAACGTTGCTGACTTGCAGAAACGTTATGG
1021-TGCTTTCGAGTATGATGTTGAAGCTGAACTCATTCGTTATAAGGAATTGGCTCAAAGACT
1081-TAAGCCATTTGTCATTGATGCTGTTGCGTTCATGTATGAAGCTTTACAAAGCAAGAAACG
1141-TATCCTTGTCGAAGGTGCTAACGCTTTGATGCTTGATTTGGACTTTGGAACTTATCCATT
1201-CGTTACCAGCTCTAACACTACTGTTGGTGGTGTTTGCACTGGTTTGGGTGTTCCTCCTCA
1261-GCGCATTGCTAACTCCATCGGTGTAGTGAAAGCTTATACAACTCGTGTAGGCGCTGGTCC
1321-TTTCCCAACGGAACAGCTCAACGAAATTGGTGACCATCTTCAAAGTGTTGGAAGAGAAGT
1381-TGGTGTTACCACCGGTCGTAAACGCCGCTGCGGCTGGCTTGATTTGGTCGTTGTCAAATA
1441-CTCTACTATGATCAATGGTTACACTTCTTTGAACCTTACCAAGCTTGACATTTTGGATGC
1501-CTTGGATGAGATCAAAGTTGCTGTTGCCTACATTATTAACGGTAAACGCATTGAAACTTT
1561-TCCTGCTGATCTTGATTCTCTTGAAGAAGCCGAGATTGTATACGAAACTTTCCCTGGTTG
1621-GAAAGTTCCTACAACAGGTATCACTCATTGGGATCAGATGCCTGAGAATGCCAAGAAATA
1681-CATAGAGTTCATTGAAAAGTTTGTTGGTGTTCCCATCACTTTCATCGGTGTTGGTCCTGG
1741-TCGTGATGAGATGTTGGTTAAGGAGTAAGTTACGATTTACAACTAGATATCCTCACCATT
1800-CTTCACTACTTTCTTTATCTAAATTTTGTTTATTATACATCTTATTTTAGCTCTTTTCCA
1861-TTTATTTTTATAGTGCTTTTCTTTTTTTCGAATAGATTCATAAAGCATATAACTC
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FIG. 3. Primary structure of the complementing gene. DNA sequences of the complementing genomic and cDNA clones revealed a gene containing two introns and coding for a protein of 434 amino acids. The underlined sequence represents the coding region as determined from the DNA sequence of the cDNA, and the two nonunderlined regions within this area represent two introns. Search of the Swiss-Prot data base with the deduced amino acid sequence revealed sequence identity with adenylosuccinate synthetases.

Sp223, DS3 was incapable of utilizing IMP as an adenine source, consistent with a defect in the adenylosuccinate synthetase gene (not shown). DS3 was also tested for Cd sensitivity (Fig. 7) and for its ability to accumulate the HMW complex (Fig. 1B). Surprisingly, the results indicate that the response of DS3 to Cd exposure is similar to that of the wild-type strain and that the disruption of *ade2* has no effect when it is the only *ade* mutation present. Because no additional phenotype was observed in DS3, the extent of the deletion outside of the adenylosuccinate synthetase-encoding region does not contribute to Cd sensitivity (see below).

Isolation of a gene capable of restoring both Cd tolerance and synthesis of the HMW PC-Cd-S^{2-} complex to LK69 suggested that this gene is the one affected in the mutant strain. To eliminate the possibility that this could be a case of extragenic suppression rather than true complementation, the *ade2* gene in the *ade6* mutant strain Sp223 was disrupted by using the same DNA fragment used in the construction of DS3 (Fig. 6). The resulting *ade2 ade6* double mutant strain DS1, like LK69, is Cd sensitive (Fig. 7). A gel filtration analysis of cell-free extracts from DS1 and Sp223 induced with Cd (Fig. 1C) demonstrates that DS1 is unable to accumulate the HMW PC-Cd-S^{2-} complex, in a fashion identical to LK69. Therefore, the isolated gene does indeed correspond to the target of the mutation in LK69.

Similarity between two segments of the purine biosynthesis pathway. The identification of *ade2* as a gene involved in the production of the HMW PC-Cd-S^{2-} complex and the realization that another mutation in the purine biosynthetic pathway was required to produce the mutant phenotype prompted a reexamination of this pathway. A graphical depiction of the purine biosynthetic pathway is shown in Fig. 8 (all abbreviations used here are defined in the legend to Fig. 8). A salient feature is the branchpoint at IMP, where the paths for synthesis of AMP and GMP diverge. Adenylosuccinate synthetase (*ade2* gene product) acts on IMP in the first step after this branchpoint. In comparison, AIR carboxylase, product of the *ade6* gene, performs in the earlier segment of the pathway. The steps of adenine synthesis before and after the IMP branchpoint in the areas of *ade6* and *ade2* are chemically similar. SAICAR synthetase (*ade7* gene product), the enzyme immediately following AIR carboxylase, catalyzes an addition reaction very similar to that performed by adenylosuccinate synthetase: addition of aspartate to a carboxylic acid group, with the simultaneous cleavage of a purine nucleoside triphosphate. In each segment, it is the product of the *ade8* gene, adenylosuccinate lyase, which catalyzes the next step in the pathway. If each of these two segments of the purine biosynthetic pathway can perform a common function essential to the formation of the HMW PC-Cd-S^{2-} complex, then both segments would have to be inoperable in order to cause the mutant phenotype. To inactivate both portions of the pathway, a single upstream mutation such as *ade6* would not be sufficient. Since an *ade6* mutant requires adenine as a growth supplement, adenine converted back into IMP through a salvage pathway via adenosine deaminase would provide a substrate for adenylosuccinate synthetase. This explanation would be compatible with the observation that genetic lesions in both segments of the pathway are required to abolish formation of the HMW PC-Cd-S^{2-} complex.

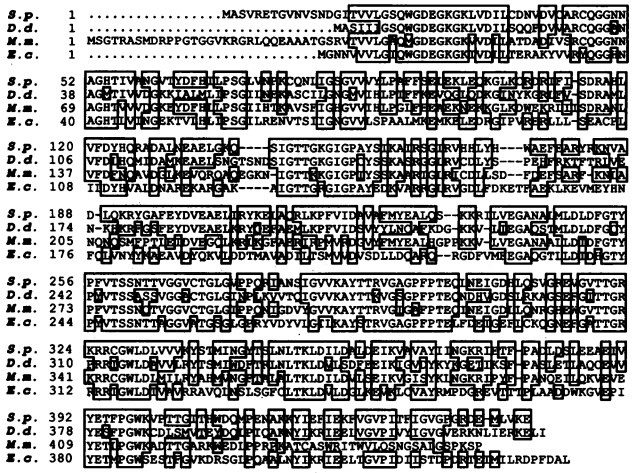

FIG. 4. Similarity of the *S. pombe* (*S.p.*) adenylosuccinate synthetase sequence to those from other organisms. The deduced amino acid sequence of the cloned gene was compared with those of the adenylosuccinate synthetase enzymes from *Dictyostelium discoideum* (*D.d.*), *Mus musculus* (*M.m.*), and *E. coli* (*E.c.*). The boxed regions represent residues identical to those in the *S. pombe* protein.

Genetic lesions affecting both segments of the purine synthesis pathway. To test the prediction that two mutations are required to block a common biochemical function provided by the above-indicated regions of the purine biosynthetic pathway, we obtained an *S. pombe* strain, B1048 (Table 1), which carries a mutation in the *ade7* gene. The *ade2* gene disruption construct was transformed into this strain as described above, and the resulting isogenic *ade7 ade2* mutant strain, DS5, was verified by Southern blot hybridization analysis for the *ade2* gene disruption (Fig. 6). Figure 7 shows that DS5 is sensitive to Cd, similar to DS1 but contrasting with the phenotype of B1048. Comparison of the gel filtration profiles from B1048 and DS5 demonstrates that blockage of purine biosynthesis at these two steps significantly reduces accumulation of the HMW PC-Cd-S^{2-} complex (Fig. 1D).

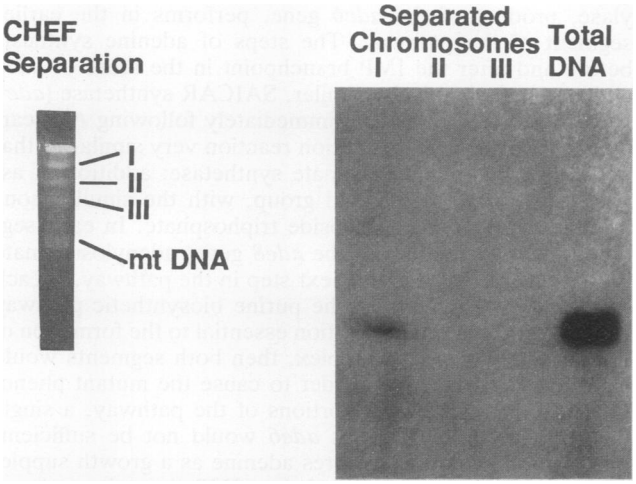

FIG. 5. The cloned gene is located on chromosome I. *S. pombe* chromosomes were separated by contour-clamped homogeneous electric field (CHEF) pulsed-field gel electrophoresis, purified from the gel, and subjected to Southern blot hybridization with a probe derived from pGS1, resulting in detection of a 2.5-kb fragment in chromosome I. Total genomic DNA was used as a control.

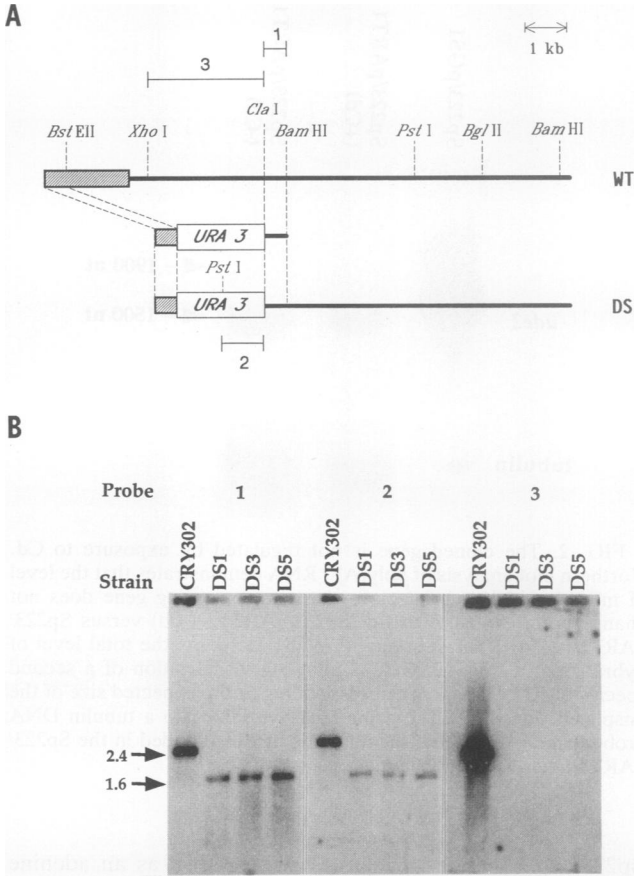

FIG. 6. (A) Structures of the DNA that complements LK69, the gene disruption fragment used to create DS1, DS3, and DS5, and the expected chromosomal region carrying the engineered deletion. A partial restriction map of pGS1 is shown, including relevant restriction sites and the complementing region (hatched box). Below this is the structure of the DNA fragment used to engineer the deletion of *ade2*, including the position of the *S. cerevisiae URA3* marker gene. Lowermost is the deduced structure of the chromosomal region surrounding an *ade2* gene deletion carrying the *URA3* insert. (B) Southern blot hybridization analysis of the three gene disruption strains used in this study (DS1, DS3, and DS5) compared with a control strain (CRY302) with a *URA3* insert elsewhere in the genome. Genomic DNA from each strain was digested with *Bam*HI, *Pst*I, and *Xho*I and separated by agarose gel electrophoresis, and a blot of the gel was hybridized to each of the three probes shown in panel A. The hybridization of probe 1 (outside the deleted gene) and probe 2 (*URA3* DNA) to the same fragment in DNA from the transformed strains and the lack of hybridization to probe 3 (covering the area of the deletion) demonstrate that the insertion has occurred in the correct location. The similarity in apparent length between the fragments from CRY302 detected by probes 1 and 3 on the one hand and probe 2 on the other results from the choice of restriction enzymes. Alternative restriction digests show the *URA3*-containing fragment in CRY302 is different from the *ade2*-containing fragment (not shown).

Correlation between production of sulfide and production of the HMW PC-Cd-sulfide complex during Cd stress. Because each of the Cd-sensitive strains does produce the LMW PC-Cd complex, we were interested in determining whether loss of the HMW PC-Cd-S^{2-} complex was accompanied by

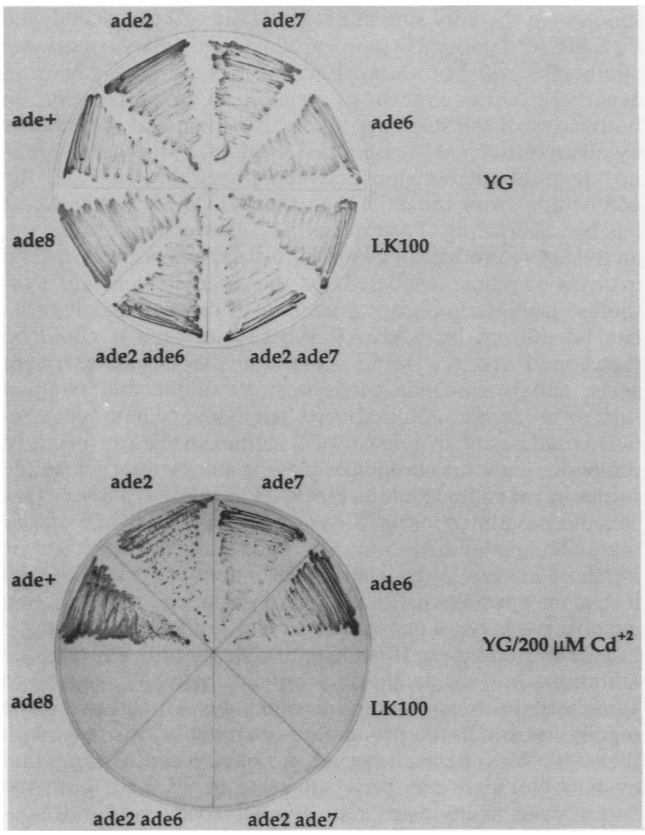

FIG. 7. Cd sensitivity of strains containing various combinations of mutations affecting adenine biosynthesis. The strains carrying the mutant alleles of the gene(s) indicated were streaked on YG medium with and without 200 μM $CdCl_2$. LK100 is another Cd-sensitive strain derived from Sp223 described elsewhere (26) and used here as a comparison.

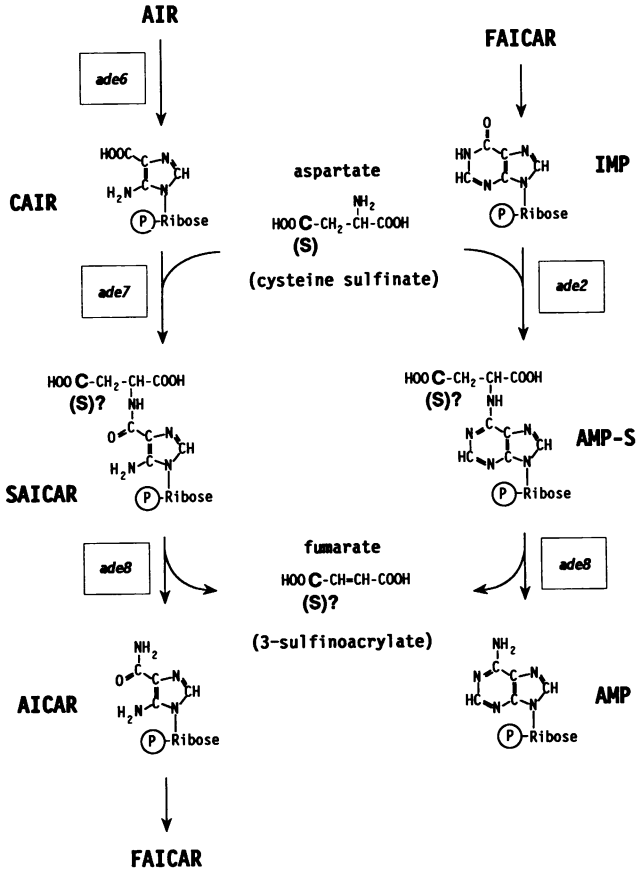

FIG. 8. A model linking the purine biosynthetic pathway with sulfur metabolism and heavy-metal tolerance. A portion of the purine biosynthetic pathway before the IMP branchpoint is shown alongside the branch after IMP. SAICAR synthetase (*ade7* product) and adenylosuccinate synthetase (*ade2* product) catalyze similar reactions which add aspartate to the growing nucleotide, and each is followed by adenylosuccinate lyase (*ade8* product), which cleaves the succinyl moiety to yield fumarate. AIR carboxylase, the step prior to SAICAR synthetase, is eliminated by *ade6.216* in Sp223 and therefore in LK69 and DS1. An additional lesion in *ade2*, whether ethyl methanesulfonate induced (LK69) or an engineered deletion (DS1), blocks both portions of the pathway, as is also the case when both *ade7* and *ade2* are mutated. One known in vivo analog of aspartate is cysteine sulfinate, produced by enzymatic oxidation of cysteine. This model proposes that addition of cysteine sulfinate to CAIR or IMP in place of aspartate would yield the indicated substituted nucleotides; if cleaved by adenylosuccinate lyase (*ade8* product), the fumarate analog 3-sulfinoacrylate would be released. Abbreviations: AIR, 5-aminoimidazole ribonucleotide; CAIR, 5-aminoimidazole-4-carboxylate ribonucleotide; SAICAR, 5-aminoimidazole-4-*N*-succinocarboxamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; FAICAR, 5-formamidoimidazole-4-carboxylate ribonucleotide; and AMP-S, adenylosuccinate.

a reduction in Cd-induced sulfide production. Acid-labile S^{2-} produced by these strains was measured after exposure to Cd, and the assays were performed on whole cells to prevent loss of S^{2-} during preparation of a cell-free extract. Table 2 shows that strains carrying a single mutation in *ade2* (DS3), *ade6* (Sp223), or *ade7* (B1048) exhibit a small reduction in sulfide production compared with that in the *ade*$^+$ control (Sp806) upon Cd exposure, whereas strains with two mutant alleles at *ade2* and *ade6* (DS1) or *ade2* and *ade7* (DS5) reduce sulfide accumulation to a level of 15 or 30%, respectively. This correlation is consistent with the possibility that Cd-induced sulfide generation is deficient in these mutants, although the data are insufficient to show a causal relationship. The sensitive mutants showed no alteration in basal sulfide levels, which are approximately 40-fold lower in the absence of Cd induction (not shown).

Involvement of adenylosuccinate lyase in Cd tolerance. If adenylosuccinate lyase is also involved in accumulation of the HMW PC-Cd-S^{2-} complex, a single lesion in *ade8* should reduce the production of sulfide as well as accumulation of the HMW complex. Gel filtration analysis shows that production of the HMW complex by the *ade8* mutant strain B23 is altered compared with that in Sp806. When exposed to Cd in rich medium for 24 h (Fig. 1F) or 30 h (not shown), B23 produces wild-type levels of the HMW PC-Cd-S^{2-} complex. With shorter induction periods, however, B23 is deficient in the accumulation of the HMW complex (Fig. 1E). In minimal medium, B23 does not accumulate the HMW complex after any length (≤30 h) of Cd induction (not shown). Table 2 shows that B23 produces sulfide at a level equivalent to those of other strains bearing single mutations in the adenine biosynthetic pathway. This reduced rate of PC-Cd-S^{2-} complex formation is accompanied by the hypersensitivity to Cd seen in the *ade2 ade6* and *ade2 ade7* double mutants (Fig. 7). Thus, a slower accumulation of the HMW

TABLE 2. Acid-labile sulfide production by *ade* strains

Strain	Relevant genotype	% Sulfide production[a] (± SD)
Sp806	*ade*$^+$	100 ± 9
Sp223	*ade6*	70 ± 5
DS3	*ade2*	68 ± 17
DS1	*ade6 ade2*	15 ± 1
B1048	*ade7*	87 ± 20
DS5	*ade7 ade2*	30 ± 5
B23	*ade8*	68 ± 25

[a] Relative to production by Sp806, which is given as 100%.

PC-Cd-S^{2-} complex is apparently sufficient to cause Cd sensitivity.

DISCUSSION

In this paper we describe the participation of the purine biosynthetic pathway in the formation of the HMW PC-Cd-S^{2-} complex, which is essential for Cd tolerance in the fission yeast. The source of the sulfide ion used to form these complexes has not been addressed; the most direct assumption has been to attribute sulfide production to the assimilatory sulfate reduction pathway and specifically to sulfite reductase or thiosulfate reductase. However, mutants LK69, DS1, DS5, and B23 have no apparent defect in this pathway, since they do not require cysteine or methionine and produce normal levels of sulfide in the absence of Cd stress. There are other known enzymatic reactions which have been shown to generate sulfide in vitro, catalyzed by cysteine desulfhydrase (cystathionase [34]: EC 4.4.1.1) and thiosulfate cyanide sulfurtransferase (rhodanese [5]: EC 2.8.1.1). The observation that formation of the sulfide-containing PC-Cd complex requires enzymes suggests that there may be a dedicated system responsible for sulfide generation during Cd stress. The surprising finding is that these enzymes belong to the purine biosynthesis pathway.

Cloning and sequencing of the *ade2* gene revealed an enzyme substantially similar in primary structure to its homologs from other organisms, with sequence identity distributed throughout the length of the protein. Verification of the identity of the *ade2* gene included the generation of the adenine auxotroph DS3 and the observation that DS3 could not be supplemented by IMP, indicating that the adenine biosynthetic pathway was blocked after this branchpoint. The Northern blot analysis shows that *ade2* mRNA accumulation does not change substantially upon exposure to Cd. This implies that if there is regulation of the gene product it is at a posttranscriptional level, but the normal role of the enzyme in an essential biosynthetic pathway may preclude extensive regulation by metals.

A model of sulfide transfer via the purine biosynthesis pathway. The identification of the purine biosynthetic pathway as an important factor in production of the HMW PC-Cd-S^{2-} complex is based on the inability of *ade2 ade6* and *ade2 ade7* (and, in minimal medium or after short inductions in rich medium, *ade8*) mutants to accumulate this complex and to tolerate Cd. It should be noted that this phenotype is seen both in rich medium and in minimal medium under conditions of adenine supplementation and cannot be ascribed simply to adenine deficiency.

The involvement of both the early and late segments of the biosynthetic pathway can be potentially explained as a function of the very similar nature of the reactions catalyzed by SAICAR synthetase (*ade7* product) and adenylosuccinate synthetase (*ade2* product). Each of these enzymes adds an aspartyl group as a means of donating an amino function. In both cases, these steps are followed by removal of fumarate by adenylosuccinate lyase (*ade8* product). Although there is no apparent relationship between these enzymes and the conversion from the PC-Cd to the PC-Cd-S^{2-} complex, it has been shown by Porter et al. (27) that cysteine sulfinate, an oxidative product of cysteine, can replace aspartate in an in vitro reaction catalyzed by the adenylosuccinate synthetase from *Azotobacter vinelandii*. If this analog substrate can be utilized by SAICAR synthetase, then it could be envisioned that the sulfur-substituted intermediates might serve either as sulfide carriers or as sulfide donors upon further reduction. Alternatively, adenylosuccinate lyase action could result in release of 3-sulfinoacrylate in place of fumarate, with this compound playing an essential role in the formation of sulfide and the HMW PC-Cd-S^{2-} complex. This scheme is outlined in Fig. 8. We as yet have no data to verify that cysteine sulfinate or any other analog substrate is involved in vivo in the production of acid-labile sulfide, and it remains possible that the reduction in sulfide accumulation we observe is not a cause, but a result, of loss of the HMW PC-Cd-S^{2-} complex. It is also possible that the effect of the mutations in the adenine biosynthetic pathway may be to alter levels of biosynthetic intermediates which have some regulatory role in the production of sulfide. Consistent with the above hypothesis, however, we have recently found that cysteine sulfinate can be a substrate in vitro for both the fission yeast adenylosuccinate synthetase and SAICAR synthetase (13a).

A role for adenylosuccinate lyase in sulfide production is implied by a lower rate of HMW PC-Cd-S^{2-} complex accumulation by an *ade8* mutant strain and by its hypersensitivity to Cd. The apparent inability of the *ade8.106* allele to abolish HMW complex accumulation might be due to a low level of adenylosuccinate lyase activity remaining in these cells. Preliminary data, however, suggest that the levels of adenylosuccinate lyase are <0.1% of those in *ade8*$^+$ strains (13a). Alternatively or additionally, the effect of *ade8.106* could be to cause accumulation of adenylosuccinate and SAICAR which might feedback inhibit the activities of their respective synthetases and prevent synthesis of the cysteine sulfinate-derived analogs. The function of adenylosuccinate lyase would in this case be indirect.

The basis of the slow accumulation of the PC-Cd-S^{2-} complex in B23 in rich medium compared with the nonaccumulation in minimal medium can be ascribed to the different sensitivities of metabolic processes to Cd intoxication. In minimal medium, the cell is generally more sensitive to Cd than in rich medium. Upon exposure to Cd, the delayed kinetics of HMW accumulation are sufficient to result in cessation of metabolic processes essential to cell growth, although formation of the HMW PC-Cd-S^{2-} complex may be less sensitive to intoxication by Cd and therefore continues even when it is too late to serve a protective function.

Involvement of purine biosynthetic enzymes in other metabolic processes has been described. Cytokinin plant growth regulators are modified purines, and enzymes of purine metabolism are involved in interconversion of cytokinin bases, nucleosides, and nucleotides (20). Mutations in the *apt* locus in *Arabidopsis thaliana*, which encodes adenine phosphoribosyl transferase, result in altered growth phenotypes because of altered cytokinin metabolism. Thus,

the involvement of purine biosynthetic enzymes in a secondary role such as Cd tolerance is not unprecedented.

Subcellular localization. Involvement of purine biosynthesis enzymes in the formation of the HMW PC-Cd-S^{2-} complex also raises a question of subcellular localization. In Cd-exposed tobacco plants, PCs and Cd are localized almost exclusively to the vacuole (39), and that also appears to be the case with the HMW PC-Cd-S^{2-} complex of *S. pombe* (26a). Assuming cytoplasmic synthesis of PCs, this localization implies vacuolar transport for one of the following: (i) PC-Cd complexes, sulfide precursors, and the enzyme(s) necessary to convert them to sulfide, (ii) PC-Cd complexes and S^{2-} (separately), or (iii) PC-Cd-S^{2-} complexes formed in the cytoplasm. High sulfide concentrations in the cytoplasm might well be toxic and this would be avoided if sulfide generation and incorporation into the PC-Cd complex were tightly coupled. It is interesting to note that in *ade6* and *ade7* mutants, a red pigment, the by-product of the buildup of a biosynthetic intermediate (AIR), is found within the vacuoles. This may indicate the presence of purine biosynthetic enzymes in the vacuoles or merely compartmentalization of the pigment as a means of preventing cytoplasmic toxicity.

Further work will be required to elucidate the involvement of adenine biosynthetic enzymes in formation of the HMW PC-Cd-S^{2-} complex. These efforts will include examination of mutants in steps downstream of those detailed here as well as attempts to detect the in vivo presence of the predicted sulfur-containing analogs of the purine intermediates. If cysteine sulfinate is involved in metal-specific sulfide generation in *S. pombe*, it might also have been recruited to serve additional roles in sulfur metabolism in other systems, given its central role in cysteine degradation in a number of organisms. The provocative possibility that enzymes of purine biosynthesis might also be involved in sulfur metabolism is worthy of further study. It is clear that conversion of the LMW PC-Cd complex to the HMW form is a process which may be dependent on numerous other elements of cellular metabolism, some of which might not be associated intuitively with the response to heavy-metal stress.

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